Ph.D Synopsis

A study on Neuroprotective potential of *in vitro* and field tissues of *Withania somnifera* using *Caenorhabditis elegans* model

Submitted
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1.0.Introduction

Since civilization began, human societies have maintained a close relationship with plants due to their magical ability to treat illness. Until date, 80% of the world's population uses plants and plant-derived products for their fundamental healthcare requirements. The quantity and quality of plants, however, have started to pose a significant obstacle to the development of herbal products. Unique sources that contribute to the therapeutic advantages of medicinal plants are their secondary metabolites which are accumulated in response to numerous biotic and abiotic stressors. (Atanasov *et al.*,2015). The synthesis of secondary metabolites is affected by UV radiation, wounding, nutritional shortages, and herbicide content in the soil. Field cultivation is difficult due to slow growth rates, the possibility of overexploitation, the accumulation of heavy metals, and microbial attacks. To develop plants that are disease-resistant, contaminant-free, and capable of rapid multiplication, plant tissue culture is an efficient technique (Altpeter *et al.,* 2016). Furthermore, *in vitro* plants grown under controlled conditions provide homogeneous production of secondary metabolites free from seasonal and environmental variance.

For the treatment of neurological illnesses including Alzheimer's disease (Dey *et al.*, 2017)) and Parkinson's disease (Zhang *et al.*,2017), herbal remedies are a promising solution. Among several medicinal plants, *Withania somnifera* (WS) holds a prominent position and is always fascinating for research because of its numerous therapeutic efficiencies. The herb is used to treat age-related illnesses and as effective adaptogens to reduce physical stress (Winston *et al.*,2007). Beyond its rejuvenating properties, it has neuroprotective effects (Kurapati *et al.*,2013) and it improves the activities of muscarinic receptors to revamp the brain's cognitive ability (Naidu *et al.,* 2006). It exhibits excellent antioxidant activity both *in vivo* and *in vitro* because of its capacity to associate and collaborate with a variety of antioxidant enzymes, such as glutathione peroxidase, monodehydroginase reductase, and peroxidase (Ahmed *et al.*,2018). The presence of steroidal lactones, specifically withanolides, is what gives the medical relevance of WS (Kaileh *et al.*, 2007). Withanolide A(WA) and withaferin A(WFA) are the two most prevalent compounds, and they are being explored more and more due to their clinically validated therapeutic significance. The WFA may pass the blood-brain barrier and is fat soluble (Swarup *et al.*,2011). WFA has also been recognized for its effectiveness in the promotion of autophagy, ubiquitin-proteasome pathway activity, anti-inflammatory effects, antioxidant benefits, and neuroprotection (Zhang *et al.*,2017). Additionally, WA has been seen

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in a number of neurological diseases, including the amyloid pathology of Alzheimer's disease, neuritis regeneration, synaptic recovery after injury, neuritis reanimation and protuberance of axonal rejoins (Baitharu *et al.*,2014).

Because of the increasing demand for bioactive withanolides, the plant has been carelessly exploited, threatening the survival of its wild population. The pharmaceutical business often employs field-grown plants that are randomly uprooted in order to meet the current demand for therapeutically effective withanolides (Mir *et al.*,2014). The extraction of withanolide from plant sources is also hampered by a long gestation period, poor growth rate, and low concentration of active chemicals. The unique accumulation patterns and structural complexity of withanolides make large-scale extraction and chemical synthesis inconvenient. So, the withanolides production from field grown plants is insufficient to meet the annual requirement (Senthil *et al.*,2015). In this scenario, to avoid this chronic shortage of WS *in vitro* cultivation serves as a possible substitute.

However, the acceptance of this approach is not justifiable without systematic scientific validation and appropriate clinical trials. Systematic validation using contemporary scientific methods and evidence is mandatory to prove the quality and efficacy of *in vitro* developed plants for medication. Hence an attempt has been made to standardize and compare the qualitative and quantitative physicochemical parameters of powder formulation developed using *in vitro* and field grown tissues of WS. We also attempted to comprehend the multidimensional animal model system *C. elegans* to investigate and compare the efficacy and safety profiles of *in vitro* and field generated WS. Further, we compared life extension and neuroprotection efficiency and some insights into the underlying mechanism of the *in vitro* and field cultivated tissues of WS. These studies have led to the hypothesis that *in vitro* culture systems of WS as an efficient neuroprotectant using the *C.elegans* model.

To validate the *in vitro* grown tissues as an alternative to traditionally used field grown roots.

The strategies followed to meet the objectives are as follows:

1. To develop *in vitro* roots and shoots in tissue culture media and compare their pharmacogenetic, and physicochemical characteristics with field grown WS
2. To compare the phytoconstituents and withanolide accumulation pattern and Antioxidant potential among tissues of WS.
3. To evaluate the difference in metal ion profile and metabolome profile of *in vitro* and field grown tissues.

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4. To investigate the life-promoting efficacy and neuroprotective efficiency among *in vitro* and field grown tissues in C.*elegans* model.

2.0. Materials and methods

2.1 Plant materials and tissue culture

We used WS shoots and roots that were developed *in vitro* (IR, IS) and in the field (FR, FS). ICAR-Directorate of Medicinal and Aromatic Plants Research, Gujarat, provided authorized 'Jawahar Aswagandh 20 seeds for *in vitro* establishment. The surface sterilization of WS seedlings was carried out as per the protocol. (Murthy *et al.*,2008). The seeds were planted on half-strength solid basal Murashige and Skoog (MS) medium fortified with 2 % sucrose and incubated at 25°C in dark. The nodal section of two-month-old, germinated plantlets was aseptically removed and inoculated on full-strength MS baseline medium fortified with 3% sucrose in 0.8 percent agar containing 6-benzyl amino purine (BAP) and kinetin (Kin). The germinated shoots were kept at 25°C for 16 hours.

Healthy bunches of shoots were kept in a suspension culture medium (3 %, 0.8 %, BAP, and Kin) and transferred aseptically to a bubble column bioreactor (Biopia, Korea) containing one litter of liquid MS media for mass production (with 3 percent sucrose, BAP and Kin). Using a mini aerator (Biopia, Republic of Korea) and a membrane filter (0.20 m), the culture is kept aseptically aerated (aeration rate 0.1 vvm). Throughout the cultivation period, the temperature was kept at 25°C. The plants were harvested after 45 days.

Matured leaves were inoculated in half strength MS medium containing IBA (indole butyric acid) and IAA (indole acetic acid) as well as 3% sucrose for root induction (Wasnik *et al.*,2009). Fresh root tips and branches weighing 500 mg (fresh weight) and measuring 2.0-4.0 cm in length were aseptically transplanted to 50 ml aliquots of full-strength liquid MS medium. These roots were kept alive for another 16 hours on an orbital shaker with constant agitation at 90 rpm and a 25°C photoperiod. The root tips and branches of roots were carefully removed from MS suspension medium and inoculated in a bubble column bioreactor containing MS baseline media enriched with 1.0 mg IBA and 0.25 mg IAA for mass multiplication of roots. After 45 days of cultivation, the roots were harvested.

2.2. Worm strains, genetics, and maintenance

The following *C. elegans* strains were used in this study: Bristol N2 (wild-type), CB1370 (*daf-2*[*e1370*]), RB759 (*akt-1*[*ok525*]), VC345 (*sgk-1*[*ok538*]), GR1307 (*daf-16*[*mgDf50*]), EU1 (*skn-1*[*zu67*]), PS3551 (*hsf-1*[*sy441*]), DA1116 (*eat-2*[*ad1116*]), TK22 (*mev-1*[*kn1*]), MQ887

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(*isp-1* [*qm150*]), CB4876 (*clk-1* [*e2519*]), RB665 (*dop-1*[*ok398*]), BZ873 (*dop-3*[*ok295*]), CF1553 (*muIs84*[*sod-3::GFP*]), CL2166 (*dvIs19*[*gst-4::GFP*]), NL5901 (*pkIs2386*[*unc-54p::alpha-synuclein::YFP+unc-119*]), and BZ555 (*egIs1*[*dat-1::GFP*]). All *C. elegans* strain were maintained and propagated on nematode growth media (NGM) agar plate pre-inoculated with *E. coli* strain OP50 as food source at 20°C. Age synchronized worm populations were obtained by treating the gravid adult worms carrying eggs with alkaline hypochlorite solution, and the collected eggs were then incubated at 20°C in M9 buffer to favor the hatching. *E. coli OP50* was cultured in the Luria-Bertani (LB) broth at 37°C overnight (Brenne,1974, Stiernagle,2006).

2.3. Preparation of powder formulation using WS tissues

The powder was formulated as per the described protocol by the Ayurvedic Formulary of India. The shade-dried tissues of WS were powdered using an electric mixer/grinder and passed through #80 sieves. For the comparative analysis, all four powders were packed individually in pre-sterilized airtight polythene bags, labelled, and stored for quality analysis (Anonymous,2008).

2.4. Pharmacogenetic study

The foreign matters and other contaminants were visualized by the unaided eye or with the help of a lens, and the total foreign substances were calculated (Zhang *et al.*,2012). The organoleptic characteristics like colour, odour, taste, total appearance, and texture of all formulations were determined as per the previously described method (Patra *et al.*, 2012). The physicochemical investigations such as total ash, water-soluble ash, acid-insoluble ash, moisture percentage, and pH range (Vikas *et al.*,2020) were carried out. Physical parameters like tap density, bulk density, angle of repose, Hausner's ratio, and Carr's index were examined using a standard protocol (Sharma *et al.*, 2019).

2.5. Microbial limit test

The microbial test included total bacterial count (TBC), total fungal count (TFC), and presence of pathogenic bacterial species such as Escherichia coli, Salmonella ebony, Pseudomonas aeruginosa, and Staphylococcus aureus (Rajput *et al.*, 2012).

2.6. Quantitative & Qualitative estimation of secondary metabolites

2.6.1. Preparation of extracts

Methanolic extraction was followed and 1gm of powdered formulation is used for the extraction. The procedure was repeated in four steps at a sample solvent ratio of 1:50. This ratio

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was fixed throughout the four steps of extraction. For every repeat, the extracts were allowed for 20 min sonication and followed by keeping them in an orbital shaker for 2 hrs at 100 rpm. The residue was filtered using Whatman No. 1 filter paper. The remaining fractions were then pooled, filtered, and vaporized to dryness by using a rotary vacuum evaporator at 125 rpm in a water bath at 40°C. The residue was dissolved in 10 mL of HPLC-grade methanol and stored at -20°C for analysis. These extracts were used for further analysis.

2.6.2. Quantitative estimation of major phytoconstituent quantification

Total alkaloid content (TAC) was investigated according to the previously mentioned method by Naik and Mishra (Naik & Mishra, 2019). The total flavonoid content (TFC) was evaluated by a procedure reported by (Lahouar *et al.*,2014). The Folin Ciocalteu method was used for the determination of total phenolic content (TPC). Total Steroid content or TSC was quantified as per the standard protocol by Naik and Mishra (Naik & Mishra, 2019).

2.6.3. Withanolide A, withaferin A withanone quantification using HPTLC

Standard of withanolide A(WA) (Chromodex (City, USA)), withaferin A(WFA) (Natural Remedies, Bangalore, India), and withaone(WT) (Chromodex (city, USA) (1.0 mg / mL) were prepared using HPLC grade methanol and stored in a refrigerator at 4°C. The working solutions were prepared from the stock by dilution with HPLC-grade methanol. HPTLC was performed on a precoated silica gel Aluminium plate (20 cm × 20 cm) 60F254 (E. MERCK, Germany). The methanolic extracts of WS were loaded to the plates as 6 mm bands, under a stream of N2 gas using the CAMAG (Switzerland) Linomat V semiautomatic sample applicator equipped with a 100 µL of Hamilton HPTLC syringe. The HPTLC plates were developed up to 80 mm using the mobile phase toluene: ethyl acetate: formic acid in the ratio of 5:5:1 in a CAMAG Twin trough glass tank. The tank was pre-saturated with the mobile phase for 30 min at room temperature (25±2ºC). The developed plate was air-dried, and the image was captured at 245 nm and 366 nm. Densitometric scanning was performed at 235 nm for WA, 231 nm for WFA and 231 nm for withnone using CAMAG TLC scanner III controlled by CAMAG CATS 4 integration software. The Rf values of the resolved spots were noted. The peak areas were evaluated using linear regression. The WA, WFA and withnone concentration in the samples was quantified using the peak area.

2.7. Determination of free radical scavenging activity

The samples extracted with 100% methanol and ascorbic acid (positive control) were dissolved

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in dimethyl sulfoxide. An aliquot (20 µL) of each of the extracted samples was introduced into a 96 well plate, and 180 µL of 100 mM DPPH solution was added. After standing for 30 min at room temperature, the absorbance was measured at 517 nm using a Microplate Reader (iMark, Biorad Japan). The DMSO was used as the blank. The free radical scavenging activity was expressed as IC50 (the concentration of the WS roots and shoot sample (µg/mL) need to scavenge 50% of DPPH) and calculated from the calibration curve developed by the free radical scavenging activity percentage of the various concentrations of the extracts (10–500 µg/mL). The IC50 of the reference antioxidant ascorbic acid (1–20 µg/mL) was calculated by the same method as described above ((Vaz *et al.*,2011).

2.8. Trace elemental fingerprinting of WS powders

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed for the evaluation metal ion profile. Concentrations of elements such as P, K, Ca, Mg, B, Cu, Fe, Mn, Mo, Zn, Cd, Ni, Pb, Hg, Cr, As, Co analysis was done using ICP-MS (Thermo Fisher iCAP RQ ICPMS, Germany). A wet digestion method was followed for ICPMS sample preparation. The finely powdered sample was digested in Anton Paar Mutiwave GO microwave digester. All the chemicals used were of supra pure or trace metal grade. 200mg of the powder sample was kept in the digestion vessel and 6 ml of nitric acid (67-70% for trace metal analysis) was added to it. After 30 minutes of pre-digestion, the vessel was closed and kept inside the digester at 453K. After completion of digestion, the samples were transferred to a volumetric tube and made up to 50 ml. The sample blank solution was prepared following the same procedure as above without adding the sample. Instrument calibration was done for all the analyzed elements by mixing the standard solutions in the required proportions.

2.9. GC-MS Analysis:

100 µl of the methanolic extracts were vacuum evaporated and the dried residues were incubated at 37^0C with 50 µl of methoxylamine hydrochloride (20mg/ml pyridine) for 2 hrs. The acidic protons formed were derivatized at 37^0C for 30 min using 50 µl of MSTFA (N-methyl N- trimethyl silyl trifluoroacetamide) and used for metabolite profiling. The gas chromatography was performed on a 0.25µm thick Rxi-5 silicon MS column 30m long and 0.25mm in diameter. The sample injection was carried out with an ion source at a temperature set to 250^0C with helium flow adjusted to 1ml min^{-1}. After an initial solvent delay time (8 min at 50^0C), the column temperature was programmed to increase at the rate of 6.5^0C min^{-1} up to 300^0C with a 5 min hold time. Each 1 µl aliquot of the derivatized extracts was injected onto

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the GC/MS system (shimadzu 2010 plus) with a split ratio of 1:10 after tuning of the system as per the manual with helium as a reference gas. The high mass resolution was improved manually to reach a maximum resolution of R_{fwhm}=2800 at 614 m/z. The mass spectra were observed between 45m/z and 650 m/z with scanning speed of 0.5s $scan^{-1}$. The interpretation of the GCMS data obtained was done using AMDIS software (version 2.62). The peaks obtained were confirmed against the two spectralibraries NIST 05 (https//:chemdata.nist.gov) and GOLM metabolome database (gmd.mpimp- golm.mpg.de). The relative amount percentage of the metabolites were extracted and subjected to Principal component analysis (PCA).

2.10. Lifespan determination assay

The age synchronized worms (10-15 worms per 60 mm plate) were aseptically transferred to NGM plates spotted without WS extracts and with extract at different pharmacological doses. During their reproductive age, the worms were transferred to fresh NGM treatment plates every day, and then every 3 to 4 days after that. In the absence of pharyngeal pumping and worm failure to respond to gentle prodding with a metal worm picker, the worms were scored dead. Censoring occurred if the worms displayed extruded inner organs, died due to internal hatching, and crawled off the plate. As mentioned, the lifespan assay was performed at least three times under identical conditions(Mohankumar *et al.*,2020)

2.11. Oxidative stress resistance

L4 stage worms were age synchronized and placed on NGM plates with and without WS extracts for 72 hours at 20°C. After that, the worms were exposed to oxidative stress. Control and treated worms (40-50 worms per experiment) were moved to fresh NGM plates containing 240 M 5-hydroxy-1,4-naphthoquinone, an intracellular ROS producer, for the oxidative stress resistance assay. After 3 hours of continuous exposure, the vitality of the worms was measured. The animals were judged dead if they did not respond to gentle probing with a metal worm picker (Devagi *et al.*,2018)..

2.12. Reporter gene expression

Transgenic strains stably expression *sod-3::GFFFP*, *ctl-1,2,3:GFP*, and *gst-4::GFP* were treated with WS extract starting from the L4 stage, as indicated in lifespan assay. After 72 h of the continuous exposure, the worms were collected, washed thrice with M9 buffer, and anesthetized with 25 mM sodium azide. The immobilized live worms (20-25 worms per experiment) were then mounted onto 3% agar padded microscopic glass slides, and the slides

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were visualized under a fluorescence microscope (B41, Olympus, Japan). The localization pattern of *daf-16::GFP* was classified into cytosolic, intermediate, and nuclear as described. Scoring of *skn-1b/c::GFP* was done as follows: none-no visible nuclear accumulation; low-only few positive nuclei showed positive expression; medium-half of the nuclei showed GFP positive; high-all nuclei showed GFP positive. The mean pixel intensity of GFP signals in worms bearing the sod-3::GFP, hsp-16.2p::GFP, and gst-4::GFP transgenes was determined using ImageJ software.

2.13. Assay for α-synuclein aggregation and neuroprotection

The strain NL5901 expressing the human gene for α-synuclein fused with a yellow fluorescent protein in body wall muscle cells was utilized to evaluate α-synuclein protein aggregation. Age synchronized worms (20-25 worms per experiment) were raised on NGM plates containing the appropriate concentration of WS extract from the L1 stage. Under the fluorescent microscope on day 6 of adulthood, worms were monitored for α-synuclein aggregation and quantified using ImageJ. Nile Red staining was used to assess changes in lipid levels in NL5901 worms, as described before (Shanmugam et al. 2017). To examine the neuroprotective effect of WS extract against 6-hydroxydopamine (6-OHDA; 25 mM)-induced selective degeneration of dopaminergic (DAergic) neurons, the BZ555 worms carrying GFP construct tagged in all 8 DAergic neurons were used. The worms were treated with either 6-OHDA or 6-OHDA/ WS extract, as described in our previous article. At the final of described treatments, the worms were imaged, and the GFP signal in the head region was quantified using ImageJ. DAergic neurons-mediated food-sensing behavior was measured according to the Bargmann method (Bargmann *et al.*,1993).

2.14. Data analysis

One-way analysis of variance (ANOVA) was used to examine the differences between the control and treated groups, followed by Bonferroni's posthoc test in SPSS 17 (IBM Corporation, USA). Regular statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, USA). Survival curves were plotted using the Kaplan-Meier survival method and analyzed by the log-rank (Mantel-Cox) test in MedCalc 14 (Ostend, Belgium). The *p*-value <0.05 was considered statically significant in all cases.

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3.0. Results

3.1. The percentage of foreign matter of FR, FS, IR and IS was less than 0.5%(w/w) which signifies their good quality and purity. The Ayurvedic pharmacopeia of India (API) permissible limits for the foreign matter of herbal powders are 0.5–2%. The organoleptic characteristic such as colour, odour, taste, appearance, and texture of formulation were also evaluated.

3.2. Further, the physicochemical analysis such as pH, Moisture percentage, total ash content, acid insoluble ash, water soluble ash was evaluated, and it is investigated that all parameters are within the Ayurvedic pharmacopeia of India (API) standard limit.

3.3 Physical characteristics like bulk density, tapped density, angle of repose, Hausners ratio and Carr's index were evaluated. The flowability of the FR and IR was observed to be poor with a tapped density of (0.548±0.01) (0.542±0.02) and bulk density (0.446 ± 0.01) (0.442±0.02) respectively. This was additionally confirmed by high values of Hausner's ratio (1.23 and 1.22) and Carr's index (18.61%, 18.75%).

3.4. Preliminary phytochemical analysis showed the presence of carbohydrates, proteins, tannins, alkaloids, phenols, flavonoids, steroids and saponins. Among the four WS tissue extracts the phenolics concentrations were observed least in FR (70.91 ± 0.53) and highest in IS (86.35± 0.72). The total steroid in root powder were ranged from FR (76.18± 0.71)> IR (64.58± 0.57) and in leaf powder FS (47.40± 0.52)> IS (42.66± 0.79). Significantly higher concentrations of alkaloid were observed in field-grown samples FR(36.00± 0.93) and FS(20.76± 0.36) and followed by IR(27.75± 0.64) and lowest in IS(19.12± 0.61).

3.5. Higher antioxidant activity was observed IS (129.16± 0.08) and IR (112.65± 0.04) than FS (98.33± 0.05) and FR (95.21± 0.06). We substantiate that a significant difference in terms of the ability to scavenge among the tissues was revealed as a percentage of inhibition on the DPPH radical. IS & IR showed maximum antioxidant potential than field-grown samples. It has been reported that a correlation between plant phenolics and free radical scavenging ability activities of plant materials. Our results are admirably supported by the reports that the phenolic content act as an indicator of total antioxidant activity.

3.6. Higher concentration of WFA was noted in FS (5.74±0.9 mg/g DW) than IS (2.76±0.8 mg/g DW). The comparatively diminutive concentration of WFA was observed in root tissues i.e., IR (0.48± 0.4 mg/g DW) and FR (1.99 ±0.4mg/g DW). Furthermore, the root tissues accumulate prodigious quantities of WA (2.83±0.4 mg/g DW) than IR (1.67±0.4 mg/g DW). Preferably shoot tissues accumulated with a lower concentration of WA i.e., IS (1.36±0.4 mg/g

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DW) than FS (0.19±0.4 mg/g DW). The WT concentration is highest in FS (3.02±0.8 mg/g DW)>IS (2.58±0.4 mg/g DW)>FR (1.29±0.7 mg/g DW)>IR (1.26±0.2 mg/g DW).

3.7. The pathogenic microorganisms such as *Salmonella sp*, *Pseudomonas aeruginosa*, *staphylococcus aureus*, and *Escherichia coli* were also absent in the four variant powders. Significantly, a moderate level of the microbial load was observed in IR and IS.

3.8. The essential and heavy metal ion fingerprinting was carried out using ICP-MS and observed that the metal concentration may vary with in the tissues of WS. It was observed that the Magnesium (Mg) concentration was significantly higher in FS (8085.9722 mg/Kg) than the WHO permissible limit (2000 mg/Kg). Further, the Chromium (Cr) was found to be higher (0.5082 mg/Kg) than that of WHO permissible limit in FR. The Mercury (Pb) content was also higher in both FR (0.3203 mg/Kg) and FS (0.5023 mg/Kg) than that of WHO permissible limit (0.001- 0.04 mg/Kg). Our results emphasized that the *in vitro* developed plants are entirely free from having metal contamination and are safe for medicinal use.

3.9. Further the profiling of bioactive compounds in the methanolic extracts of FS, FR, IR and IS were carried out using GC-MS. The curation of GC-MS data with AMDIS software and retrieval of the peaks with NIST and GOLM library showed the presence of total of 33 metabolites related to withanolide biosynthesis. The metabolites reported include 13 organic acids, 10 amino acids, 3 sugars, 3 alcohols and 4 other compounds.

3.10. Among the 13 organic acids, the FS showed high content of propanoic acid, linoleadic acid, oxalic acid, myristic acid, linolenic acid, fumaric acid and valeric acid, butanoic acid, levulinic acid, oxalic acid whereas field roots accumulated high levels stearic acid, succinic acid and stearic acid.

3.11. The amino acids such as proline, glycine, aspartic acid, L.valine and glutamine was found to be higher in FS compared to the other tissue extracts. Both FS and FR samples showed higher levels of amino acids asparagine and glutamic acid which were present in very trace quantities in IS tissues. Significantly higher amount of tryptophan observed in all samples except IR.

3.12. The sugars such as mannose, galactos and glycerol were found to be higher in FS and glucose is comparatively higher in FR, IR. were present in very trace quantities in FS, IS. Whereas aziridine levels is higher in FR, IR. Phytol content is comparatively low in IS, IR and higher in field grown plants FR, FS. The percentage of myo-inositol and putresin which were only in trace quantities in FR, IR and higher in FS,IS.

3.13. The survival and reproduction of *C. elegans* were affected differently by acute exposure to different dosages of IR, FR, IS, FS; 0, 10, 20, 40, 80, 160, 320, and 640 g/mL.

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The results showed that IR and IS extracts did not adversely affect normal survival, reproduction rate, and progeny production. Among the tested doses, FR extract at 640 µg/mL and FS extract at 320 and 640 µg/mL were found to be toxic and significantly ($p<0.01$) reduced the percentage survival of wild-type worms when compared to that of unexposed worms. In addition, 160-640 µg/mL FS extract significantly affects the overall reproduction rate and progeny development. Hence, the toxic concentrations of WS extracts were excluded, and non-toxic concentrations were used to study their life-promoting efficacy in *C. elegans*.

3.14. It was observed that 160 µg/mL of IR and IS, and 320 µg/mL of FR being the highly effective concertation and they could extend the mean lifespan of *C. elegans* by 21.07% (20.97±0.46 days; $p<0.0001$), 21.41% (22.94±0.39 days; $p<0.0001$), and 12.14% (19.86±0.43 days; $p<0.0001$), respectively. Other tested concentrations of IR (20, 40, 80, and 320 µg/mL) and IS (80, and 320 µg/mL) significantly extend the mean lifespan of *C. elegans* ($p<0.05$, $p<0.01$), and they also exhibited hormesis-like, dose-dependent biphasic effect. FR extracts displayed a concentration-dependent effect, while FS extracts did not show any effects on the lifespan of *C. elegans* at any of the concentrations tested ($p>0.05$). It is crucial to note that the lifespan extension induced by IR and IS extracts is significantly higher than the effects that are produced by FR extract at higher concentrations. These results suggest that 160 µg/mL of IR and IS, and 320 µg/mL of FS are more effective in promoting the mean lifespan of *C. elegans*

3.15. Genetic screening to identify the potential mechanism underlying WS extracts-mediated lifespan extension using specific *C. elegans* strains deficient in the IIS pathway genes (*daf-2*[*e1370*], *akt-1*[*ok525*], *sgk-1*[*ok538*], *daf-16*[*mgDf50*], *skn-1*[*zu67*], *hsf-1*[*sy441*]), mitochondrial electron transport chain complex genes (*mev-1*[*kn1*], *isp-1* [*qm150*], *clk-1* [*e2519*]), and DR genes (*eat-2*[*ad1116*]). The results showed that IR extract significantly extended the lifespan of *sgk-1*, *clk-1 sgk-1*, *hsf-1*, *eat-2* and mev-1 mutant worms, indicating that IR extract does not require these genes for lifespan extension. Simultaneously, IR extract fails to extend the lifespan of worms carrying a loss of function mutation in *daf-2*, *akt-1*, *skn-1*, and *isp-1*, showing that *daf-2*, *akt-1*, *skn-1*, and *isp-1* are required for the lifespan extension observed in IR extract treated worms. In addition, the lifespan of *daf-16* mutant worms was marginally increased by the IR extract, indicating the partial requirement. The IS extract treatment significantly extended the lifespan of *eat-2* and *mev-1* worms. However, no corresponding lifespan extension was observed in *daf-2*, *akt-1*, *sgk-1*, *daf-16*, *skn-1*, *hsf-1*, *isp-1*, and *clk-1* worms. These results clearly indicated that IS extract acted on the IIS pathway and mitochondrial respiration but independently of the DR-like mechanism. In addition, FR extracts

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significantly prolong the lifespan *sgk-1*, *hsf-1*, *eat-2*, *mev-1*, *isp-1*, and *clk-1* as seen in wild-type worms, while no significant changes in the lifespan were observed in *daf-2*, *akt-1*, *daf-16*, and *skn-1*. These data strongly indicated that the FR extract required the IIS pathway, but not mitochondrial function and DR for lifespan extension in *C. elegans*. In lifespan experiments we found that all WS extracts notably ($p<0.0001$) extended the lifespan of *mev-1* mutant worms under standard conditions. The succinate dehydrogenase cytochrome b560 subunit, an integral membrane protein that is a subunit of mitochondrial respiratory chain complex II, is encoded by mev-1 in *C. elegans* (succinate-CoQ oxidoreductase). MEV-1 is essential for oxidative phosphorylation, and its absence resulted in aberrant energy metabolism and increased intracellular ROS, resulting in a shorter lifespan. These observations suggest that WS extracts could prolong the lifespan of short-lived *mev-1* mutant worms through their potent antioxidant activity and their involvement in the endogenous detoxification pathway. These results are mostly in accordance with previous findings.

3.16. The percent survival of wild-type worms pretreated with IR, IS and FR extracts were significantly increased by about 77.69%, 83.07%, and 66.44%, respectively, compared to the untreated control groups (34.74%). In daf-16 and skn-1 mutant worms, the improved tolerance to oxidative stress is lost, but not in dop-3 mutants. These findings revealed that functional DAF-16 and SKN-1 were mandatory for stress protection in all WS extracts, but not DOP-3. dop-3 is a homolog of mammalian D2 dopamine receptors that regulates the basal slowing response and stress resistance in well-fed animals. IR and IS extract treatment fail to protect the worms deficient in *dop-1*, suggesting the involvement of DOP-1 in IR and IS extract-mediated stress protection. *dop-1* encodes a D1-like dopamine receptor and regulates mechanosensory behaviors. Conversely, FR extract significantly enhanced the survival rate of *dop-1* mutants under oxidative stress conditions, as seen in wild-type worms. This result showed that *dop-1* is not required for oxidative stress resistance following FR extract treatment. Overall, the stress-protecting properties of IR and IS extract were dependent on *daf-16*, *skn-1*, and *dop-1*; however, FR extracts were only dependent on *daf-16* and *skn-1*. These results are consistent with the lifespan experiments. Increased expression of stress-protective genes is linked to higher survival under oxidative stress conditions.

3.17. The effects of WS extracts on the expression of stress-responsive genes *viz*., *gst-4*, *sod-3*, and *ctl-1,2,3* using transgenic reporter strain stably expressing *gst-4*::*GFP*, *sod-3*::*GFP*, and *ctl-1,2,3*::*GFP* transgene. These genes are direct transcription targets of DAF-16 (*sod-3* and *ctl-1,2,3*) and SKN-1 (*gst-4*) transaction factors and offer conserved protection against various

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stresses. As a result, the expression levels of *gst-4*::*GFP* and *sod-3*::*GFP*, were significantly upregulated by all three WS extracts ($p<0.05$ or $p<0.01$), but *ctl-1,2,3*::*GFP* expression was upregulated by IS extract alone ($p<0.05$) . These results suggest that enhanced activation of DAF-16 and SKN-1 downstream target genes (*gst-4*, *sod-3*, and *ctl-1,2,3*) by WS extracts suppresses the oxidative stress and prolongs the lifespan of *C. elegans*.

3.18. The control and FR, FS and IR treated NL5901 worms (animals expressing α-synuclein in body wall muscle cells) were examined for α-synuclein aggregation levels at day 6 adulthood. All three WS extracts significantly reduced the fluorescence intensity of α-synuclein::YFP in NL5901 worms. It was observed that the aggregation of α-synuclein was decreased to 36.68%, 68.01%, and 30.25% in NL5901 worms treaded with IR, IS, and FR extracts, respectively. Interestingly, the efficacy of IS on α-synuclein aggregation was more potent than that of the positive control EGCG and reduced the aggregation by 46.84% ($p<0.01$ vs. EGCG). No significant changes in α-synuclein protein aggregation were observed between EGCG, IR, and FR treatments. In addition, WS extracts (IR, IS, and FR) significantly expanded the lifespan of NL5901 worms by 23.89%, 43.68%, and 24.93%, receptively.

3.19. Since the pathogenesis of Parkinson's disease is associated with altered lipid and fatty acid levels due to the excessive accumulation of misfolded α-synuclein, therefore, we measured the lipid content in NL5901 worms using Nile Red staining. The results showed that NL5901 worms exhibited dramatically lower lipid content (53.27%) compared to wild-type worms, however, treatment with WS extracts restored lipid levels throughout the body of worms which expressing the α-synuclein. The effect of IS extract on lipid levels in NL5901 worms was comparable to that of EGCG. These data strongly confirmed that WS extracts have the potential to reduce Parkinson's and associated pathologies.

3.20. Neuronal viability was analyzed by quantifying the expression of GFP transgene labeled in all eight DAergic neurons of BZ555 *C. elegans*. 6-OHDA, a catecholaminergic neurotoxin, was used as an inducer of experimental PD models. 6OHDA enters DAergic neurons via dopamine-active transporter and disrupts neuronal functions. The 6-OHDA exposure reduced the GFP intensity by up to 60.98% in BZ555 worms compared to unexposed control groups, indicating their neurotoxic properties. Furthermore, when the worms were treated with IR,IS, and FR extracts, the neurons displayed enhanced GFP expression in all DAergic neurons. Moreover, treatment with IR, IS, and FR extracts increased the basal slowing response to food to 0.59±0.25, 0.72±0.11, and 0.58±0.13 in worms exposed to 6-OHDA

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(0.17±0.20). Untreated control group worms exhibited 0.79±0.08 as the slowing rate to food. It has been well established that dopamine-mediated neural networks control the slowing response to food in *C. elegans*.

3.21. The life-promoting efficacy of WS extracts in worms exposed to 6-OHDA was measured. The results showed that 6-OHDA exposure reduced the mean lifespan by 53.39% (7.72±0.27 days); in contrast, treatment with IR, IS, and FR extracts extended the lifespan of worms intoxicated with 6-OHDA to 14.62±0.36, 15.97±0.36, and 14.93±0.37 days, respectively. These results indicated that WS extracts not only protect neurons from degeneration but also extend the lifespan of worms.

4.0. Conclusion

We presented numerous lines of evidence regarding the effectiveness of the *in vitro* cultivated WS on the health and life expectancy of *C. elegans* over FR and IR. We compared the mechanisms underlying the beneficial effects of field cultivated and *in vitro* grown WS supplementation by using gene-specific mutants and green fluorescent protein-tagged worms. The efficacy of WS extracts to prevent α-synuclein aggregation, its associated pathologies, and its capability for neuroprotection are studied in Parkinson's disease modeled worms. Our findings highlighted the *in vitro* grown WS is equally bioactive as field grown roots and a remarkable neuroprotectant, implying that it might be used to treat Parkinson's disease. The *in vitro* cultivation also exhibits rapid multiplication of plantlets with strong antioxidant potential, uniformly distributed metabolites, and plantlets that are free from microbial and metal contamination. Hence, they can be used as an alternative to field-grown plants.

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